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13. ABSTRACT (Maximum 200 words)

Biomembranes are involved in some of the most basic cellular processes. These include ion transport, energy transduction, nerve transmission, sensory detection and cellular communication. The design of a new class of materials based on biomembrane components holds promise in diverse areas including optical recording media, chemical sensors, nanometer lithography, energy transducers and enzyme catalysis. However, future progress in these areas will depend on the development of new methods for elucidating the molecular basis for biomembrane function, self-assembly into higher order structures and modifying membrane components for biomaterial applications. In this project, we proposed to address these problems by developing powerful new methods based on molecular genetics and advanced biophysical techniques which have the capability to modify and characterize biomembranes on a molecular level. Key among these techniques will be site-directed non-native amino acid replacement (SNAAR) along with the related technique of site-directed isotope labeling (SDIL). These approaches when combined with FTIR spectroscopy will provide a powerful method for determining the role of individual amino acid residues in the functioning of a protein and in self-assembly as well as providing a new dimension in protein engineering, enabling the replacement of native amino acid residues with custom designed residues.

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Enclosure 1

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Sincerely,

Dr. Kenneth Rothschild

Enclosure 3

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The research agreement identified below is under consideration for additional funding. The contemplated funding, resulting in an extension to the performance period of the agreement, is consistent with the terms and conditions of the agreement. Before a decision can be made to provide the additional funding, the following information is needed: (i) an accounting of costs incurred to date and (ii) a projection of financial needs for the period

Final Report

New Biophysical and Genetic Methods for Advanced Material Development

DAAL03-92-G-0172

July 1, 1992-June 30, 1999

P.I.-Kenneth J. Rothschild
Boston University

1. Foreword

This project has led to important progress in the area of biomolecular engineering. One innovation is the development of powerful methods for probing protein conformational changes by combining Fourier transform infrared (FTIR) difference (*1*) spectroscopy with methods for site directed isotope labeling (SDIL) and site-directed replacement of non-native amino acid residues (SNAAR). This new field of tRNA mediated protein engineering (TRAMPE) is likely to impact many areas of biotechnology including biomaterial design, drug discovery and molecular diagnostics. (*2*). A second area of research in this project involved the design and synthesis of a variety of photocleavable reagents which can be introduced into proteins and nucleic acids using synthetic and enzymatic methods. This work has led to a variety of novel applications involving photocleavage in several critical areas of biotechnology including biomolecular purification, rapid DNA sequencing, multiplex detection of mutations in genes (genotyping), and cell separation and analysis. It is anticipated that additional progress will occur as technology developed under this project become better known and reagents and integrated systems incorporating this technology become commercially available.

2. Table of Contents

3. Statement of the Problem

Biomembranes are involved in some of the most basic cellular processes. These include ion transport, energy transduction, nerve transmission, sensory detection and cellular communication. The design of a new class of materials based on biomembrane components holds promise in diverse areas including optical recording media, chemical sensors, nanometer lithography, energy transducers and enzyme catalysis. However, future progress in these areas will depend on the development of new methods for elucidating the molecular basis for biomembrane function, self-assembly into higher order structures and modifying membrane components for biomaterial applications. In this project, we addressed these problems by developing powerful new methods based on molecular genetics and advanced biophysical techniques which have the capability to modify and characterize biomembranes on a molecular level. Key among these techniques were site-directed non-native amino acid replacement (SNAAR) along with the related technique of site-directed isotope labeling (SDIL). These approaches when combined with FTIR spectroscopy provide a powerful method for determining the role of individual amino acid residues in the functioning of a protein and in self-assembly as well as providing a new dimension in protein engineering, enabling the replacement of native amino acid residues with custom designed residues.

4. Summary of Most Important Results

We summarize below some of the key areas of progress for this project:

1. Development of a method for in vitro production, refolding of functional bacteriorhodopsin in lipid membranes.

Bacteriorhodopsin (bR) is an integral membrane protein which functions as a light-driven proton pump in *Halobacterium halobium* (also known as *Halobacterium salinarum*). The cell-free synthesis of bR in quantities sufficient for FTIR and NMR spectroscopy and the ability to selectively isotope label bR using aminoacylated suppressor tRNAs would provide a powerful approach for studying the role of specific amino acid residues. However, no integral membrane protein has yet been expressed in a cell-free system in quantities sufficient for such biophysical studies. We reported (*3*) the cell-free synthesis of bacterioopsin, its purification, its refolding in polar lipids from *H. halobium*, and its regeneration with all-*trans*-retinal to yield bacteriorhodopsin in a form functionally similar to bR in purple membrane. Functionality of the cell-free expressed bR is established using static and time-resolved absorption spectroscopy and FTIR difference spectroscopy.

2. Incorporation of Site-Directed Isotope Labels to study bacteriorhodopsin conformational changes during its photocycle

Insight into integral membrane proteins function is presently limited by the difficulty of producing three-dimensional crystals. In addition, X-ray structures of proteins normally do not provide information about the protonation state and structural changes of individual residues. We reported in this work (*4*) the first use of site-directed isotope labeling and Fourier transform infrared (FTIR) difference spectroscopy to detect structural changes at the level of single residues in an integral membrane protein. Two site-directed isotope labeled (SDIL) tyrosine analogues of bacteriorhodopsin were produced which exhibit normal activity. FTIR spectroscopy shows that out of 11 tyrosines, only Tyr 185 is structurally active during the early photocycle and may be part of a proton wire.

3. Creating by molecular engineering a redirected proton pathway in bacteriorhodopsin

Light-driven proton pumping in bacteriorhodopsin involves deprotonation of the retinylidene Schiff base during M formation and reprotonation during N formation as key steps. This study (*5*) reported on the spectroscopic characterization of the bacteriorhodopsin mutant Tyr-57 → Asp (Y57D). The results reveal that although formation of the M intermediate and Schiff base deprotonation is blocked, the mutant still exhibits a significant level of light-driven proton translocation. The photocycle of Y57D involves formation of K and L intermediates accompanied by the normal chromophore isomerization and changes in the hydrogen bonding of Asp-96 and Asp-115. However, an additional Asp residue deprotonates during formation of the L intermediate along with a transmembrane alpha-helical structural change that normally occurs upon N formation. We postulate that proton transport in Y57D occurs through a redirected pathway that does not involve the deprotonation of the Schiff base. Chromophore isomerization, which normally results in the transfer of a proton from the Schiff base to Asp-85, instead causes the deprotonation of Asp-57 in Y57D, most likely through an interaction involving Asp-212. This deprotonation of Asp-57 causes the release of a proton into the extracellular medium. Reprotonation of Asp-57 occurs through the Schiff base reprotonation pathway, which consists of a hydrogen-bonded network of residues spanning from Asp-96 to Asp-212. The results also indicate that the transmembrane alpha-helical structural changes observed during N formation [Rothschild, 1993 #6486]do not require deprotonation of Asp-96 or of the Schiff base.

4. Proton Pathway of BR probed during the early photocycle using SDIL

Fourier transform infrared difference spectroscopy has been used extensively to probe structural changes in bacteriorhodopsin and other retinal proteins. However, the absence of a general method to assign bands to individual chemical groups in a protein has limited the application of this technique. While site-directed mutagenesis has been successful in special cases for such assignments, in general, this approach induces perturbations in the structure and function of the protein, thereby preventing unambiguous band assignments. A new approach has recently been reported [Sonar, 1994 #6498]which involves cell-free expression of bacteriorhodopsin and site-directed isotope labeling (SDIL). We have now used this method to re-examine bands assigned in the bR → M difference spectrum to tyrosine residues (*6*). Our results show that out of 11 tyrosines in bR, only Tyr 185 is structurally active. This work further demonstrates the power of SDIL and FTIR to probe conformational changes at the level of individual amino acid residues in proteins.

5. Escherichia coli initiator tRNA: structure-function relationships and interactions with the translational machinery

In an earlier study by one of the co-P.I.'s of this project (U.L. RajBhandary) it was shown that the sequence and (or) structural elements important for specifying the many distinctive properties of *Escherichia coli* initiator tRNA are clustered in the acceptor stem and in the anticodon stem and loop. This work (7) briefly describes this and reviews the results of some recently published studies on the mutant initiator tRNAs generated during this work. First, we have studied the effect of overproduction of methionyl-tRNA transformylase (MTF) and initiation factors IF2 and IF3 on activity of mutant initiator tRNAs that are defective at specific steps in the initiation pathway. Overproduction of MTF rescued specifically the activity of mutant tRNAs defective in formylation but not mutants defective in binding to the P site. Overproduction of IF2 increased the activity of all mutant tRNAs having the CUA anticodon but not of mutant tRNA having the GAC anticodon. Overproduction of IF3 had no effect on the activity of any of the mutant tRNAs tested. Second, for functional studies of mutant initiator tRNA in vivo, we used a CAU $\rightarrow$ CUA anticodon sequence mutant that can initiate protein synthesis from UAG instead of AUG. In contrast with the wild-type initiator tRNA, the mutant initiator tRNA has a 2- methylthio-N6-isopentenyl adenosine (ms2i6A) base modification next to the anticodon. Interestingly, this base modification is now important for activity of the mutant tRNA in initiation. In a miaA strain of E. coli deficient in biosynthesis of ms2i6A, the mutant initiator tRNA is much less active in initiation. The defect is specifically in binding to the ribosomal P site.

6. Design and synthesis of a photocleavable biotin

While the strong biotin-avidin interaction has been widely used for the detection of biomolecules, its irreversibility complicates their isolation. We reported in this work (8) the synthesis of a photocleavable biotin derivative (PCB) which eliminates many limitations of existing methods. This reagent contains a biotin moiety linked through a spacer arm to a photocleavable moiety, which reacts selectively with primary amino groups on any substrate. In experiments using [leucine]-enkephalin as a model substrate, we show that PCB retains its high affinity toward avidin/streptavidin and allows rapid (< 5 min) and efficient (> 99%) photorelease of the substrate in a completely unaltered form. Photocleavable biotins should be useful in numerous applications involving the isolation of proteins, nucleic acids, lipids, and cells. Importantly, it can be used also as a means to isolate nascent proteins produced in cell-free reactions.

7. Probing conformational changes in bacteriorhodopsin during the M to N transition using a combination of ATR FTIR difference spectroscopy and SDIL-Evidence for a major conformational change involved in proton transport.

The largest secondary structural change occurs in the bacteriorhodopsin (bR) photocycle during the M-->N transition. In this work site-directed isotope labeling (SDIL) and attenuated total reflection Fourier transform infrared (ATR-FTIR) difference spectroscopy were used to investigate this conformational change (*9*). L-Tyrosine containing a 13C isotope at the carbonyl carbon was selectively incorporated at Tyr 57, Tyr 147, and Tyr 185 by SDIL. This involves the cell-free expression of bR in the presence of *Escherichia coli* suppressor tRNA (CUATyr) aminoacylated with L-[1-13C]Tyr. ATR-FTIR difference spectroscopy reveals that of the 11 tyrosines, only the peptide carbonyl group of Tyr 185 undergoes a significant structural change during the bR-->N transition. Along with other spectroscopic evidence, this result suggests that the Tyr 185-Pro 186 region of the protein is structurally active and may function as a hinge which facilitates the tilt of the cytoplasmic portion of the F-helix in bacteriorhodopsin during the M-- >N transition.

8. Site-Directed Isotope Labeling and FTIR Spectroscopy: The Tyr 185/Pro 186 peptide bond of bacteriorhodopsin Is perturbed during the primary photoreaction

In this work, (*10*) we have used FTIR-SDIL to probe protein conformational changes which occur in bacteriorhodopsin (bR), a light-driven proton pump from *Halobacterium salinarum*, during the primary photochemical reaction (bR→K). While it is known that this initial step involves an all-trans to 13-cis isomerization of the retinylidene chromophore', little is known about the interactions which occur between the retinal and protein during this step or how these interactions eventually facilitate proton transport[8]. We have probed the response to chromophore isomerization of three specific tyrosine amide carbonyl groups residing in the retinal binding pocket (Tyr 57, 83 and 185)[9] as well as Tyr 147 outside of this pocket. Our results show that out of the eleven tyrosine residues in bR only the amide carbonyl group of Tyr 185 is perturbed by chromophore isomerization.

9. Probing the conformation and orientation of a model membrane protein: Phospholamban.

Phospholamban is a 52 amino acid residue membrane protein involved with the regulation of calcium levels across sarcoplasmic reticulum membranes in cardiac muscle cells. The N-terminal 30 amino acid residues of the protein are largely hydrophilic and include two sites whose phosphorylation is

thought to dissociate an inhibitory complex between phospholamban and Ca2+ ATPase. The C-terminal 22 amino acid residues are largely hydrophobic, anchor the protein in the membrane and are responsible for Ca2+ selective ion conductance. Specific interactions between the transmembrane domains stabilize a pentameric protein complex. We have obtained circular dichroism (CD), transmission Fourier transform infrared (FTIR) and attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of the full-length protein and have compared these results to those from a 28 residue peptide that includes the transmembrane domain. (*11*) Both proteins reconstituted into phospholipid membranes are largely alpha-helical by CD and FTIR. Polarized ATR-FTIR measurements show that both the cytosolic and transmembrane helices are oriented perpendicular to the membrane plane with a tilt of 28 (+/- 6) degrees with respect to the membrane normal. This tilt angle is in close agreement to that calculated from a model for the transmembrane domain of phospholamban suggested by mutagenesis and molecular modeling. Phosphorylation does not significantly change the secondary structure or orientation of the protein. The pentameric complex is modeled as a left-handed coiled-coil of five long helices (40 (+/- 3) residues) that extend across the membrane from the lumenal carboxy terminus to the phosphorylation site in the cytoplasm. The helix bundle forms a perpendicular ion pore that may begin at a distance (17 to 29 A) from the membrane surface. Based on the above, we propose a mechanism by which phospholamban regulates Ca2+ levels across membranes that takes into account both its selective ion conductance and inhibitory association with the Ca2+ pump.

10. Utilization of SDIL to probe local regions in Membrane Proteins-A model study on Phospholamban.

Phospholamban is a 52-amino acid residue membrane protein that regulates Ca(2+)-ATPase activity in the sarcoplasmic reticulum of cardiac muscle cells. The hydrophobic C-terminal 28 amino acid fragment of phospholamban (hPLB) anchors the protein in the membrane and may form part of a Ca(2+)-selective ion channel. In this work (continuation of 7) (*12*) we have used polarized attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy along with site-directed isotope labeling to probe the local structure of hPLB. The frequency and dichroism of the amide I and II bands appearing at 1658 cm-1 and 1544 cm-1, respectively, show that dehydrated and hydrated hPLB reconstituted into dimyristoylphosphatidycholine bilayer membranes is predominantly alpha- helical and has a net transmembrane orientation. Specific local secondary structure of hPLB was probed by incorporating 13C at two positions in the protein backbone. A small band seen near 1614 cm-1 is assigned to the amide I mode of the 13C-labeled amide carbonyl group(s). The frequency and dichroism of this band indicate that residues 39 and 46 are alpha-helical, with an axial orientation that

is approximately 30 degrees relative to the membrane normal. Upon exposure to 2H2O (D2O), 30% of the peptide amide groups in hPLB undergo a slow deuterium/hydrogen exchange. The remainder of the protein, including the peptide groups of Leu-39 and Leu-42, appear inaccessible to exchange, indicating that most of the hPLB fragment is embedded in the lipid bilayer. By extending spectroscopic characterization of PLB to include hydrated, deuterated as well as site-directed isotope-labeled hPLB films, our results strongly support models of PLB that predict the existence of an alpha-helical hydrophobic region spanning the membrane domain.

11. Design and synthesis of efficient photocleavable biotin linkers for nucleic acid.

We reported in this work the design, synthesis and evaluation of a non-nucleosidic photocleavable biotin phosphoramidite (PCB-phosphoramidite) which provides a simple method for purification and phosphorylation of oligonucleotides (*13*). This reagent introduces a photocleavable biotin label (PCB) on the 5'-terminal phosphate of synthetic oligonucleotides and is fully compatible with automated solid support synthesis. HPLC analysis shows that the PCB moiety is introduced predominantly on full-length sequences and is retained during cleavage of the synthetic oligonucleotide from the solid support and during subsequent deprotection with ammonia. The full-length 5-PCB-labeled oligonucleotide can then be selectively isolated from the crude oligonucleotide mixture by incubation with immobilized streptavidin. Upon irradiation with 300-350 nm light the 5'-PCB moiety is cleaved with high efficiency in <4 min, resulting in rapid release of affinity- purified 5'-phosphorylated oligonucleotides into solution. 5'-PCB- labeled oligonucleotides should be useful in a variety of applications in molecular biology, including cassette mutagenesis and PCR. As an example, PCB-phosphoramidite has been used for the synthesis, purification and phosphorylation of 50-and 60mer oligonucleotides.

12. Probing the biophysical basis of membrane protein folding using transmembrane fragments of BR.

In order to characterize the thermodynamic constraints on the process of integral membrane protein folding and assembly, we have conducted a biophysical dissection of the structure of bacteriorhodopsin (BR), a prototypical alpha-helical integral membrane protein. (*14*) Seven polypeptides were synthesized, corresponding to each of the seven transmembrane alpha-helices in BR, and the structure of each individual polypeptide was characterized in reconstituted phospholipid vesicles. Five of the seven polypeptides form stable transmembrane alpha-helices in isolation from the remainder of the tertiary structure of BR. However, using our reconstitution protocols, the polypeptide corresponding to the F helix in BR does not form any stable secondary structure in reconstituted vesicles, and the

polypeptide corresponding to the G helix forms a hyperstable beta-sheet structure with its strands oriented perpendicular to the plane of the membrane. [The polypeptide corresponding to the C helix spontaneously equilibrates in a pH-dependent manner between a transmembrane alpha-helical conformation, a peripherally bound non-helical conformation, and a fully water soluble conformation; the conformational properties of this polypeptide are the subject of the accompanying paper: Hunt et al. (1997) Biochemistry 36, 15177-15192.] Our observations suggest that the folding of alpha-helical integral membrane proteins may proceed spontaneously. However, the preference for a non-native conformation exhibited by two of the polypeptides suggests that the formation of some transmembrane substructures could require external constraints such as the links between the helices, interactions with the rest of the protein, or the involvement of cellular chaperones or translocases. Our results also suggest a strategy for improving the thermodynamic stability of alpha-helical integral membrane proteins, a goal that could facilitate attempts to overexpress and/or refold them.

13. Discovery of a pH controlled insertion of a transmembrane membrane protein fragment from BR.

A question of fundamental importance concerning the biosynthesis of integral membrane proteins is whether transmembrane secondary structure can insert spontaneously into a lipid bilayer. It has proven to be difficult to address this issue experimentally because of the poor solubility in aqueous solution of peptides and proteins containing these extremely hydrophobic sequences. We have identified a system in which the kinetics and thermodynamics of alpha-helix insertion into lipid bilayers can be studied systematically and quantitatively using simple spectroscopic assays (*15*). Specifically, we have discovered that a 36- residue polypeptide containing the sequence of the C-helix of the integral membrane protein bacteriorhodopsin exhibits significant solubility in aqueous buffers free of both detergents and denaturants. This helix contains two aspartic acid residues in the membrane-spanning region. At neutral pH, the peptide associates with lipid bilayers in a non-helical and presumably peripheral conformation. With a pKa of 6.0, the peptide inserts into the bilayer as a transbilayer alpha-helix. The insertion reaction proceeds rapidly at room temperature and is fully reversible.

14. Development of compositions and methods for incorporating photocleavable labels and linkers into nucleic acid.

We report (*16*) the design and evaluation of two non-nucleosidic photocleavable aminotag phosphoramidites. These reagents introduce a photocleavable amino group on the 5'-terminal phosphate of synthetic oligonucleotides. The 5' photocleavable amino group enables introduction of a variety of amine-reactive markers onto synthetic oligonucleotides as well as immobilization on activated solid

supports. The photocleavable bond on the 5'-phosphate can then be selectively cleaved by near-UV illumination, thereby enabling release of the marker or detachment of the oligonucleotide from a solid support. The preparation of photocleavable conjugates with biotin, digoxigenin and tetramethylrhodamine are described. In the case of biotin, a conjugate was used in a high sensitivity hybridization assay as a photocleavable probe for a complementary sequence immobilized on beads. It is also demonstrated that the 5'-PC-amino group can be used as an affinity tag for photocleavage-mediated affinity purification and phosphorylation of synthetic oligonucleotides in conjunction with activated supports. Such 5'-PC-amino labeled oligonucleotides should be useful in a variety of applications in molecular biology including multiple non-radioactive probing of DNA/RNA blots, affinity isolation and purification of nucleic acids binding proteins, diagnostic assays requiring release of the probe-target complex or specific marker, cassette mutagenesis and PCR. They will also enable the spatially-addressable photorelease of the probe-target complexes or marker molecules for diagnostic purposes.

15. Development of photoactive probes for SDIL/SNAAR:

The fluorescent non-native amino acids, hydroxycoumarinyl alanine (HCA) and hydroxynaphthyl alanine (HNA), have been synthesized for the site-specific non-native amino acid replacement in proteins. The photophysics of HCA and a model compound, 4-methyl umbelliferone (4-MU), have been studied both in aqueous solution and in poly(methacrylic acid) polymer environment. The results suggest that HCA will have strong fluorescence and its fluorescence spectra will provide information about the micro-environment after replacement in a protein. Computer modeling and molecular dynamics suggest that bacteriorhodopsin (bR) modified by replacing Tyr-185 with HCA or HNA will maintain the same structure and hydrogen-bond as the native protein.

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Applications Conference: 4th Annual 1994 IEEE Mohawk Valley Section., editors. SUNY Institute of Technology at Utica/Rome. 326-331

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6. List of Personnel who worked on Project and Advanced Degree Earned

Name	Role	Degree Earned
Kenneth J. Rothschild	P.I.	None
Uttam L. RajBhandary	Co-PI	None
Guilford Jones	Co-PI	None
Sanjay Sonar	Postdoc.	None
Jerzy Olejnik	Postdoc	None
Sergey Mamaev	Postdoc	None
Chan-Ping Lee	Postdoc	None
Jun-Suk Jang	Postdoc	None
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Nilam Patel	Graduate Student	B.S., M.S.
Xiao-Mei Liu	Graduate Student	Ph.D.
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Matthew Rothman	Graduate Student	M.S.
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Rashmi Pandey	Undergraduate	B.S.
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Edyta Olejnik	Technical Staff	none
Natalia Mamaev	Technical Staff	none

7. Report of Inventions by Title

1. "Methods for the Detection and Isolation of Proteins
2. "Photocleavable Agents and Conjugates for the Detection and Isolation of Biomolecules,"

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